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Antigen processing for MHC presentation via macroautophagy

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Abstract

Macroautophagy has recently emerged as an important catabolic process involved not only in innate immunity but also in adaptive immunity. Initially described to deliver intracellular antigens to MHC class II loading compartments, its molecular machinery has now also been described to impact the delivery of extracellular antigens to MHC class II loading compartments through the *non-canonical* use of the macroautophagy machinery during LC3-associated phagocytosis (LAP)(1). Therefore, in pathological situations (viral or bacterial infections, tumorigenesis) the pathway might be involved in shaping $CD4^+$ T cell responses.

In this chapter we describe three basic experiments for the monitoring and manipulation of macroautophagic antigen processing towards MHC class II presentation through the *canonical* pathway. Firstly, we will discuss how to monitor autophagic flux and autophagosome fusion with MHC class II loading compartments. Secondly, we will show how to target proteins to autophagosomes in order to monitor macroautophagy dependent antigen processing via their enhanced presentation on MHC class II molecules to $CD4^+$ T cells. And finally, we will describe how macroautophagy can be silenced in antigen presenting cells, like human monocyte-derived dendritic cells (DCs).

Key words:

Macroautophagy, $CD4^+$ T cells, MHC class II loading compartment

1. Introduction

Since antigen processing for MHC presentation to T cells relies on cellular proteolytic systems, like lysosomes for MHC class II and proteasomes for MHC class I presentation (2, 3), cellular transport pathways that deliver proteins to these catabolic machineries can contribute to immune surveillance by T cells. Autophagy is no exception. It describes at least three pathways, by which cytoplasmic constituents gain access to lysosomal degradation. These are chaperone-mediated, micro- and macroautophagy (4). Both chaperone-mediated and microautophagy rely on substrate recognition by HSC70 chaperones, and proteins selected for it carry a pentameric recognition sequence (5, 6). For chaperone-mediated autophagy HSC70 docks to LAMP2a in the lysosomal

membrane and the substrates are then translocated across this membrane with the help of luminal HSP70 chaperones. For microautophagy, substrate carrying HSC70 molecules attach to late endosomal membranes, which then bud into the endosomal lumen, and endosome-lysosome fusion then delivers this cargo for degradation. While chaperone-mediated and microautophagy degrade only soluble proteins, macroautophagy is able to deliver larger protein aggregates and whole cell organelles for lysosomal degradation (7). For this purpose, a cup-shaped isolation membrane forms around the autophagic cargo to finally engulf it completely in a double membrane surrounded autophagosome. This autophagosome then fuses with lysosomes for degradation of its content and the inner autophagosomal membrane. This process requires more than 30 so-called autophagy related genes (atgs), which were originally described in yeast, but have now also been identified in higher eukaryotes (8). Of these, we will only discuss two complexes in this chapter, which are relevant for the described experiments, while a more complete description has been given in a recent review (9). The two Atg complexes that are primarily targeted for macroautophagy monitoring and manipulation, center around the two ubiquitin-like molecules Atg8 with its main mammalian homologue LC3, and Atg12. Atg12 gets conjugated to Atg5 by the two E1- and E2-like enzymes Atg7 and 10. The resulting conjugate then associates with Atg16L1 at the outer autophagosomal membrane. This complex catalyzes ligation of Atg8 to phosphatidylethanolamine (PE), a phospholipid in the autophagome membrane. Prior to this coupling, Atg8 gets cleaved by Atg4 to liberate a C-terminal glycine residue, which conjugates to PE, and is activated by the E1- and E2-like enzymes Atg7 and Atg3. Atg8 is thought to facilitate lipid fusion during isolation membrane generation (10) and substrate recruitment (11). While the Atg5/12/16L1 complex and Atg8 are recycled from the outer autophagsomal membrane upon vesicle completion, Atg8 stays attached to the inner autophagosomal membrane and gets degraded with it after fusion with lysosomes. This can be monitored for autophagosome visualization and turn-over. Furthermore, primarily Atg5 and 7 are targeted to inhibit macroautophagy.

Immunologists are mainly interested in macroautophagy for three reasons. It can degrade intracellular pathogens (12), modulates pathogen detection (13) and it assists in antigen processing for MHC presentation (14). Macroautophagy facilitates MHC

presentation to T cells mainly via three pathways. It directly imports antigens into MHC class II loading compartments (15), it augments antigen packaging in antigen donor cells for efficient cross-presentation (16, 17) and through the non canonical LAP pathway it modulates exogenous antigen delivery for MHC class II presentation (18). Intracellular antigen processing via macroautophagy might account for 20-30% of natural MHC class II ligands that are derived from cytosolic or nuclear proteins (19), including self-protein derived peptides that are involved in positive and negative selection of $CD4^+$ T cells in the thymus (20). In addition to self-proteins, few viral and bacterial antigens have been reported to be processed via macroautophagy for MHC class II presentation (21-24). Here we will focus on the classical macroautophagy pathway, while the monitoring of antigen presentation through the *non-canonical* LAP pathway has been reviewed elsewhere (1).

For the monitoring of the canonical pathway of autophagic antigen processing for MHC class II presentation we will describe three experiments. **Firstly,** we will discuss how the tandem GFP-RFP-Atg8/LC3 construct can be used to monitor autophagic flux and autophagosome fusion with MHC class II loading compartments (**Fig. 1**). **Secondly,** we will show how Atg/LC3 fusion proteins can be used to monitor macroautophagy dependent antigen processing via their enhanced presentation on MHC class II molecules to $CD4^+$ T cells (**Fig. 2**). And finally, we will describe how macroautophagy can be silenced in antigen presenting cells, like human monocyte-derived dendritic cells (DCs), via Atg specific siRNA (**Fig. 3**). These basic experiments allow for the monitoring and manipulation of macroautophagic antigen processing towards MHC class II presentation. They characterize this specific antigen processing pathway and the level of macroautophagy in antigen presenting cells in general.

2.Materials

2.1. Cell Culture

2.1.1. Cell lines (*see* Note 1):

1. Human embryonic kidney epithelial cell line, HLA-$DR4^+$ HEK 293 (obtained from Dr. Rong-Fu Wang, Houston, Texas).

2. Human lung epithelium cell line A549 (obtained from Dr. Thomas Moran, New York, NY).
3. Human melanoma cell line M199 (obtained from from Dr. Jean-Francois Fonteneau, Nantes, France).
4. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM, Gibco,) with 10% fetal bovine serum (FBS), 2 mM glutamine, 110 μg/ml sodium pyruvate, and 2 μg/ml gentamicin (Gibco).

2.1.2. Human dendritic cells

1. PBMCs are isolated from leukocyte concentrates (Zurich Blood Center) by density-gradient centrifugation on Ficoll-Hypaque. $CD14^+$ monocytes/macrophages are isolated by positive magnetic cell separation (Miltenyi Biotec) and differentiated to monocyte-derived DCs with IL-4 and GM-CSF and matured with appropriate stimuli as described in **Section 3.1.2**.
2. Dendritic cell culture medium: RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen), and 2 μg/ml gentamicin (Gibco).

2.1.3. Cytokines

1. Recombinant human interferon-gamma (IFN-γ) (Peprotech) is reconstituted in sterile H_2O + 0.1% human serum albumin (HSA, Sigma) to a concentration of 10 ng/μl and frozen aliquots are kept at -20ºC.
2. Recombinant human interleukin-4 (IL-4) (Peprotech) is reconstituted to a concentration of 20 ng/μl and frozen aliquots are kept at -20ºC.
3. Granulocyte–macrophage colony-stimulating factor (GM-CSF) (Invitrogen) is reconstituted to a concentration of 20 ng/μl and frozen aliquots are kept at -20ºC

2.2. Antibodies

2.2.1. Primary antibodies

1. Maturation of monocyte-derived DCs is monitored by flow cytometry using anti-CD11c, -CD83, -CD86, -HLA-DR and HLA-ABC antibodies (BD Biosciences).
2. Expression of MHC class II molecules:
    a) The mouse monoclonal HLA-DR/DP/DQ-specific hybridoma IVA12 (ATCC). The hybridoma is grown in RPMI-1640 with 10% FBS, 2 mM

glutamine, 2 μg/ml gentamicin and supernatant is harvested by spinning cells down at 300 x *g* for 10 min. Supernatant is filtered through a 0.2-μm filter and stored at 4°C. Use at a 1:10 dilution.

b) A mouse monoclonal antibody against human LAMP2 (lysosomal-associated membrane protein 2) (clone H4B4) antibody (eBioscience) is used at 0.5μg/ml.

c) A mouse monoclonal antibody against HLA-DR molecules (clone L243) (eBioscience) is used at 1 μg/ml.

3. Expression of LC3 is monitored with:
   a) Rabbit polyclonal anti-LC3 antibody (MBL) use at 1/1000.
   b) Mouse monoclonal anti-LC3 antibody (Clone 5F10) (Nanotools) use at 1/500.

2.2.2. Secondary antibodies.

1. Alexa fluor-555 conjugated goat anti-mouse: to be used at 1/500 dilution.
2. Alexa fluor-488 conjugated goat anti-rabbit: to be used at 1/500 dilution.
3. Alexa fluor-647 conjugated rat anti-mouse: to be used at 1/500 dilution.
4. Alexa fluor-647 conjugated goat anti-mouse: to be used at 1/500 dilution.

2.3 Expression of NY-ESO-LC3 Fusion Construct and the RFP-GFP-LC3 tandem construct by transient transfection

1.Plasmids DNA

- NY-ESO/LC3 fusion construct is obtained by inserting the human Atg8/LC3 cDNA sequence (genebank entry NM_022818) and the human NY-ESO-1 cDNA sequence NM_001327.1 into the mammalian expression vector pEGFP-C2 (BD, Biosciences)
- NY-ESO-1 (pCMV6-XL4) (Origene) expression vector encoding for the human NY-ESO-1 is used in parallel as a control.
- The fusion mRFP-GFP tandem fluorescent LC3 (tfLC3) construct is obtained from Dr. Tamotsu Yoshimori (Osaka, Japan).

For better transfection results, maxipreps of the different DNA plasmids should be prepared and DNA should be eluted in sterile DPBS. DNA concentration and purity is

determined by OD260/OD280 absorption spectroscopy in a spectrophotometer. OD260/OD280 ratio should be greater than 1.8. DNA and is stored in aliquots at −20ºC.

2. Transient transfection medium: cell culture medium without gentamicin *(see* **Note 2**).

3. Transfecting reagent: Lipofectamine 2000 (Invitrogen).

4. Transfecting medium: OptiMEM-I (Gibco) without Phenol Red.

2.4. Immunocytochemistry and Confocal Microscopy to Analyze MHC Class II Compartments

1. Circular 1.5-mm cover slips (Fisher) (*see* **Note 3**).
2. 70% (v/v) ethanol
3. Sterile DPBS.
4. Chloroquine (CQ) (Sigma) the stock is prepared in water at a concentration of 20 mM, and frozen aliquots are stored at -20C, the working concentration is 50μM (i.e. dilute the stock to 1/400)
5. Fixative solution 4% Paraformaldehyde
6. Permeabilization solution: 0.1% (v/v) Triton-X 100 in PBS.
7. Blocking buffer: PBS, 10% normal goat or donkey serum (NDS or NGS from Sigma), 0.1% saponin (Calbiochem) (*see* **Note 4**).
8. Staining buffer: PBS, 5% normal goat or donkey serum (NDS or NGS from Sigma), 0.1% saponin (Calbiochem)
9. Washing buffer: 1X PBS.
10. DAPI nucleic acid staining solution: Prepare a 5 mg/ml stock of 4,6-diamidino-2-phenylindole (DAPI, Invitrogen-Molecular Probes) in ddH2O and store in aliquots at -20ºC. Dilute 1:10000 in PBS to freshly prepare a working solution.
11. Mounting medium: Prolong Gold Antifade Reagent (Invitrogen-Molecular Probes).
12. Confocal laser scanning microscope, using a high N.A. oil immersion lens (e.g., 63x/1.4 N.A.)
13. ImageJ software

2.5. MHC Class II Presentation Assay Using a NY-ESO-1 -Specific CD4$^+$ T-Cell Clone

1. A NY-ESO-1 specific CD4$^+$ T-cell clone for the 157-170 epitope of this tumor antigen (obtained from Dr. Jean-Francois Fonteneau, Nantes, France) is cultured in RPMI-1640 with 8% pooled human serum (PHS, Mediatech Inc.), with 450 U/ml recombinant human IL-2 (Peprotech), 2 mM glutamine, 2 μg/ml gentamicin in round-bottom 96-well plates (*see* **Note 5**).
2. Recombinant human IFN-γ (Peprotech) as in **Section 2.1.3**.
3. Coculture medium: RPMI-1640 with 5% PHS, 2 mM glutamine and 2 μg/ml gentamicin.
4. As positive control the following stimuli is used: specific NY-ESO-1 peptide (1 mM stock in 10% DMSO, for T-cell stimulation dilute 1:1000 in coculture medium) or phyto-hemagglutinin (PHA-L, Sigma, 1 mg/ml stock, for T-cell stimulation dilute 1:1000 in coculture medium).

2.6. IFN-γ ELISA to Analyze Secretion of IFN-γ by NY-ESO-1 specific clonal CD4$^+$ T Cells

1. High-protein-binding 96-well ELISA plates (e.g., Maxisorp, Nunc).
2. ELISA kit for human IFN-γ (Mabtech, Nacka Strand). A 10 μg/ml stock of human recombinant IFN-γ provided with the kit is prepared and frozen in aliquots at -20^{o}C. A freshly thawed aliquot should be used for each experiment.
3. coculture medium as in **Section 2.5.**
4. Blocking buffer: PBS, 1% BSA (Sigma).
5. Washing buffer: PBS, 0.05% Tween-20.
6. Incubation buffer: PBS, 0.1% BSA, 0.05% Tween-20.
7. TMB peroxidase substrate solution (Sigma)
8. Stop solution: 1 N sulfuric acid.

2.7. Silencing macroautophagy in dendritic cells

1. Stealth siRNA: All Stealth™ siRNA duplexes (Invitrogen) are dissolved to a final concentration of 100 μM according to the manufacturer's instructions. Scrambled

siRNA are ordered for each target sequence. In our case, we used siRNA targeting the Atg16L1 sequence: Sense- 5'GAG UUG UCU UCA GCC CUG AUG GCA G3' and Anti-Sense-5'CUG CCA UCA GGG CUG AAG ACAACUC3'

2. Electroporation medium: Opti-MEM without phenol red (Invitrogen).
3. 4mm electroporation cuvette (Biorad).
4. Electroporator: ECM830 Square Porator TM

3. Methods

3.1. Cell Culture

3.1.1. Cell lines

1. A549, M199 and HLA-DR4$^+$ HEK 293 cells are maintained in 100mm plates until they approached confluence.
2. To split cells, monolayers are washed once with 5 ml of DPBS and incubated with 2 ml of 0.025 % trypsin/EDTA solution at 37ºC for 2–3 min to detach cells. To set up new maintenance cultures, the cells are re-plated at 1/5 for M199 and A549, and at 1/10 for HLA-DR4$^+$ HEK 293 and fresh culture medium is added. These cultures approach confluence after 2–3 days.
3. To induce expression of the MHC class II antigen processing machinery in the M199 cell line, cells are cultured with 200 units/ml of IFNγ, for 48 hours. At least 50% of cells express MHC class II on their cell surface after this treatment.

3.1.2. Monocyte-derived dendritic cells

1. PBMCs are isolated from leukocyte concentrates by density-gradient centrifugation on Ficoll/Hypaque.
2. CD14$^+$ monocytes are isolated from PBMCs by positive magnetic cell separation (Miltenyi Biotec), then plated in 6 well plates at a density of 2-3x10^6 cells per well, in a final volume of 3 ml, and cultured for 5 days in dendritic cell medium (RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen)). GM-CSF and (IL)-4 are added to the culture on days 0, 2, and 4, each at a final concentration of 20 ng/ml.

3. On day 5 immature DCs are collected after pipetting up and down each well at least 20 times with a 1ml pipette. Remaining adherent cells should not be collected.
4. Cells are then counted and plated in a 12 well plate at a concentration of 1 million cells/ml and matured with the appropriate stimuli (in our case influenza infection).
5. Matured dendritic cells are collected at day 6 and their maturation phenotype checked by FACS analysis.

3.2. Expression of NY-ESO-LC3 or the tandem RFP-GFP-LC3 Fusion Construct by Transient Transfection

1. To set cells up for transfection, cells are detached as described in **Section 3.1.1. step 2** and plated in 6-well tissue culture plates in transient transfection medium at a density of 2-3x10^5 cells/well (*see* **Note 2**).
2. The next day, cultures should be about 70–80% confluent.
    a) For each well to be transfected, 2.5 μg plasmid DNA are diluted in 250 μl OptiMEM-I medium and mixed by vortexing briefly.
    b) In a separate tube, 5 μl lipofectamine 2000 are diluted in 250 μl OptiMEM-I medium and vortexed briefly.
    c) Both tubes are incubated for 5 min at room temperature.
    d) Both solutions are combined and briefly vortexed, followed by incubation for 20 min at room temperature to allow formation of DNA–lipofectamine complexes.
3. Complexes are then added in a dropwise manner to the culture medium of cells in the 6-well plate and incubated at 37ºC for 4 hours.
4. After 4 hours, the complex-containing medium is replaced with fresh culture medium, and cells are cultured for 18–20 h at 37ºC (*see* **Note 6**).
5. Twenty-four hours post transfection, cells are ready to be split onto microscopy cover slips for localization analysis by confocal immunofluorescence microscopy (see **Section 3.3**) or for use in MHC presentation assays (see **Section 3.4**).

3.3. Immunocytochemistry and Confocal Microscopy to analyse autophagosomes flux and MHC Class II Compartment

1. Round 1.5-mm microscopy cover slips are placed into 24-well tissue culture plate. Eight extra cover slips are used for control stainings (*see* **Note 7**).
2. Cover slips are then sterilized by washing once with 70% ethanol and twice with sterile DPBS. Any traces of ethanol are removed by completely aspirating ethanol and wash solutions with a vacuum suction flask.
3. Cells, transfected with the mRFP-GFP tandem fluorescent LC3 (tfLC3) construct (see **Section 3.2.**), are trypsinized and plated onto sterilized cover slips in cell culture medium, at a density of 200000 cells/well. Two wells of each sample should be plated, so that cells could be analyzed with and without chloroquine (CQ) treatment or Toll like receptor stimulation.
4. During the last 24h of the culture, one set of cells are treated with 50 μM CQ to prevent degradation of the tfLC3 construct by lysosomal proteases, or/and the cells are stimulated with the TLR agonist or infected with the pathogen of interest depending on the experimental set up (in our case influenza infection for 24 hours). Leave at least 2 wells of cells untreated as a control (*see* **Note 7**).
5. Cells are then washed once in PBS (0.5 ml/well) and fixed in 4% paraformaldehyde (PFA, 200 μl/well) for 15 min at room temperature (*see* **Note 8**).
6. Then cells are washed once in PBS (0.5 ml/well) and permeabilized in 0.1% Triton X-100 (200 μl/well) for 5 min.
7. Next, cells are washed once in PBS (0.5 ml/well) and blocking buffer (200 μl/well) is added for 30 min.
8. The primary antibodies are diluted in blocking buffer and added to cells (200 μl/well) for 45 min at room temperature or for longer periods (up to overnight) at 4ºC. *Co-staining of LC3 and MHC class II compartment is possible by using the anti-LC3 rabbit antibody and one of the mouse antibodies specific for MHC class II compartment.*
9. Then cells are washed three times in washing buffer (0.5 ml/well) and incubated for 5 min each time.

10. The secondary antibodies are diluted in blocking buffer and added to cells (200 µl/well) for 30 min.
11. Secondary antibody solutions are then aspirated and DAPI nucleic acid stain is added for 1 min (200 µl/well). Afterwards, cells are immediately washed.
12. Cells are then washed once in PBS and cover slips are mounted on microscopy slides by inverting them onto a drop of mounting medium on a microscopy slide, up to three cover slips per slide. Next, cover slips are carefully press down, excess mounting medium is aspirated and slides dry at room temperature in the dark (*see* **Note 9**). Afterwards, slides could be stored in the dark at 4ºC for several months.
13. Slides are then analyzed with a confocal laser scanning microscope, using a high N.A. oil immersion lens (e.g., 63x/1.4 N.A.). Excitation at 405 nm elicited DAPI fluorescence (blue emission), excitation at 488 nm Alexa 488 fluorescence, or GFP (green emission), and excitation at 543 nm RFP or Alexa 555 fluorescence (red emission). Image J Software or Imaris Software are used to overlay the different fluorescence channels and to quantify colocalization (*see* **Note 10**). Two experiments are shown as an example in **Figure 1**: An experiment with influenza virus matrix protein 2 (MP2) transfection of A549 cells expressing the tandem RFP-GFP-LC3 construct is shown in **Fig. 1A**. In **Fig.1B** an example of MHC class II staining of HEK 293 cells that were infected with influenza A virus is shown.

3.4. MHC Class II Presentation Assay Using a NY-ESO-1 Specific $CD4^+$ T-Cell Clones

1. For MHC class II presentation assays, HLA-$DR4^+$ HEK 293 or M199 cells transfected with the two different NY-ESO-1 constructs (see Section 2.3) in a 6-well format are used, as described in **Section 3.2**. M199 cells are treated with 200 U/ml IFN-γ for 48 hours to initiate expression of the MHC class II antigen processing machinery.
2. Any traces of IFN-γ are removed from cells by washing cell monolayers three times in RPMI-1640 medium. Then cells are trypsinized to prepare a cell suspension, washed once in coculture medium and counted with a

hemacytometer. Cell suspensions are prepared in co-culture medium at three different cell concentrations ($2x10^5$, 10^5 and $6.67x\ 10^4$ cells/ml) (*see* **Note 11**).

3. NY-ESO-1 specific clonal $CD4^+$ T cells are collected from 96-well culture plates, washed once in coculture medium and counted. The cell concentration is adjusted to $2x10^6$ cells/ml.
4. Cocultures of T cells and target cells are set-up in duplicates (2 wells/condition) in a 96-well round-bottom plate. Per well, 50 µl of T-cell suspension (10^5 cells/well) and 100 µl of the different target cell suspensions ($2x10^5$, 10^5 and $6.67x10^4$ cells/ml) are added. This results in effector to target (E:T) ratios of 5, 10 and 15. As a positive control, clonal T cells are stimulated with the cognate NY-ESO-1 peptide (1 µM) or PHA-L (1 µg/ml). As a negative control, clonal T cells are exposed to coculture medium alone.
5. Cells are then cultured overnight (18–24 h) at 37 ºC.

3.5. IFN-γ ELISA to analyze secretion of IFN-γ by NY-ESO-1 specific clonal $CD4^+$ T cells

1. One day prior to ELISA, high-protein-binding ELISA plates are coated with a primary anti-IFN-γ antibody (1-D1K, included in IFN-γ ELISA kit), diluted 1:500 in PBS, 100 µl/well. Plates are then incubated overnight at 4 ºC.

2. The next day, the plates are washed two times with PBS (200 µl/well) and blocked with blocking buffer (200 µl/well) for 1 h at room temperature.

3. An aliquot of IFN-γ standard (10 µg/ml) is thawed and serial dilutions are prepared in coculture medium (prepare 2000, 1000, 500, 250, 125, 62.5, 31.25 pg/ml standards, at least 300 µl each).

4. To make sure that IFN-γ secreted by T cells is homogeneously distributed in culture supernatants, supernatants are mixed by pipetting up and down with a multichannel pipet and cells are then pelleted by centrifugation of plates at 300*g* for 5 min.

5. With a multichannel pipet, 120 µl of supernatant are carefully removed from each well and transferred to a new 96-well plate.

6. ELISA plates are washed four times with washing buffer.

7. The coculture supernatants or IFN-γ standards (100 µl/well) are then added and incubated for 2 hours at room temperature. The remaining 20 µl of supernatant are frozen at –20°C in case ELISA has to be repeated on diluted supernatants (*see* **Note 13**).

8. Plates are then washed as in **step 6** and 100 µl/well of secondary antibody (7-B6-1-biotin, provided in ELISA kit) are added, diluted 1:1000 in incubation buffer. An incubation time of one hour at room temperature follows.

9. Again, plates are washed as in **step 6** and 100 µl/well of streptavidin-HRP (provided in ELISA kit) are added, diluted 1:1000 in incubation buffer. Another hour at room temperature is observed as incubation time.

10. As in **step 6** plates are washed again and 100 µl/well of TMB peroxidase substrate is added. The plates are incubated until blue reaction product has sufficiently developed, then the reaction is stopped by adding 100 µl/well of Stop solution.

11. Optical density is measured at 450 nm (OD450) in an ELISA plate reader and OD450 values are converted into IFN-γ concentration in pg/ml, using the IFN-γ standards (*see* **Note 12**). An example of the produced results is shown in **Fig. 2**.

3.6. Silencing Macroautophagy in Monocytes-derived Dendritic Cells

1. Electroporation of immature monocyte-derived DCs with siRNA targeting Atg16L1 or Atg5 or scrambled siRNA is performed *at day 4* (*see* **Note 13 and Note 14**). For this purpose, immature DCs are collected after pipetting up and down each well at least 20 times with a 1ml pipette, and then washed once in RPMI without serum. Remaining adherent cells should not be collected.

2. Cells are adjusted to a final concentration of 3-4x10^6 immature DCs/ in 200 µl Opti-MEM medium without phenol red, and transferred to an electroporation cuvette. The cells are kept at 4°C until the electroporation step.

3. One nmol of the siRNA of interest (Atg16L1 in our case or the scrambled control siRNA) is added to the cells directly in the 4 mm cuvette on ice just prior to electroporation.

4. The cells are then electroporated in an ECM830 Square Porator TM with a unique square wave pulse of 500V 0.5ms.

5. Cells are then plated at a density of 1 million cells/ml in a 12 well plate in R2 medium supplemented with GM-CSF and IL-4.
6. Maturation of these immature DCs is performed the next day, *at day 5,* using the maturation stimuli of interest (in our case influenza A infection).
7. At day 6 matured dendritic cells are collected for analysis. Maturation phenotype and viability of the cells are checked by FACS analysis (*see* **Note 15**).
8. Immunocytochemistry and microscopy are then performed as described in **Section 3.3.**

4. Notes

1. Cell lines are chosen based on their ability to up-regulate MHC class II expression upon IFN-γ treatment and their MHC class II expression matching the restriction of the used T cell clone. The M199 cell line carries HLA-DP4 and the used $CD4^+$ T cell clone is restricted by both DR4 and DP4 molecules. Therefore, HLA-$DR4^+$ HEK293 cells are also used in some experiments.
2. For transfection with lipofectamine 2000, cell culture medium should not contain any antibiotics. Therefore, gentamicin is omitted for the transfection.
3. Non-adherent cells are cytospinned on 1.5 mm coverslips that are pre-coated with L-polylysine (Sigma).
4. Normal donkey or goat sera are used as blocking reagents because secondary antibodies are derived from donkey or goat. For secondary antibodies from different species 5% normal serum from that species should be used as blocking reagent. Alternatively, 5% BSA could be employed as blocking solution.
5. T cell clones are expanded in RPMI-1640 + 8% PHS + 150 U/ml rhIL-2 (Chiron) + 1 μg/ml PHA-L (Sigma), in 96-well U bottom plates. Up to $1x10^4$ clonal T cells/ well, 10^5 irradiated allogeneic irradiated PBMCs/well and 10^4 irradiated LCLs/well are added as feeder cells. T cell clones should not be used prior to 2 weeks after re-expansion to allow the T cells to rest after proliferation and avoid high background reactivity.
6. The transfection medium should be changed after 4 hours not only to improve the viability of the transfected cells, but also to prevent autophagosome accumulation due to an effect of the lipofectamine 2000 transfection reagent.

7. For correct interpretation of results, the following control stainings should be included:
    a) Primary and secondary antibodies should be replaced with blocking buffer. These stainings should be completely negative.
    b) Primary antibody should be replaced with blocking buffer, but secondary antibodies should be used. Background from secondary antibodies should be low. If the background turns out to be too high, the concentration of the secondary antibodies must be lowered.
    c) Untransfected cells and cells that are not treated with IFN-γ or with TLR/maturation stimuli are used as controls.
    d) Single-labeling of cells should be performed and signal bleed-through into non-specific neighbouring fluorescence channels should be checked (red channel for Alexa488 labeling and green channel for Rhodamine-Red X or Alexa555 labelings). There should be no signal in these channels.
8. From the fixation step onwards, cells could be handled outside sterile biosafety cabinets on a laboratory bench. Vacuum suction flasks can be used to change solutions, but the plastic tip of suction device should be exchanged between different solutions (e.g., antibodies). All incubation steps are done at room temperature, unless noted otherwise.
9. Prolong Gold antifade mounting medium (Invitrogen-Molecular Probes) should be allowed to dry at room temperature, in the dark, overnight. During this time, the mounting medium solidifies and its refractive index increases. Sealing of cover slips with nail polish is not necessary for Prolong Gold, but is recommended for other, water-based mounting media.
10. If a confocal microscope is not available, alternatively slides can be analyzed with a conventional wide-field fluorescence microscopes with a motorized z-stage. To remove out-of-focus light and accurately analyze colocalization of fluorochromes, z-stacks subsequently have to be deconvoluted using a deconvolution software.
11. The optimal number of target cells per clonal T cell may vary, depending on the T-cell clone and the type of target cell. Therefore it is recommended to try a range

of different effector and target cell numbers, ranging from 10^4 to $2x10^5$ T cells/well and 10^3 to 10^5 target cells/well.

12. In case IFN-γ levels in supernatants exceed the linear range of the ELISA (approximately 20–1000 pg/ml), frozen supernatants are diluted 1:10 in coculture medium and the ELISA assays are repeated.
13. In case of ATG proteins, it is optimal to perform the knock down 48 hours prior to the analysis, since the half life of these protein is usually >24 hours.
14. In order to discriminate between LAP and the canonical macroautophagy pathway selected siRNAs could be used. Molecules required exclusively for the canonical pathway are ULK1, FIP200, ATG13, Ambra1, WIPI2 and ATG14. In parallel Rubicon and NOX2 are only activated in the LAP pathway. All the other components of the formation of autophagosomes are common to both pathways, in particular the ATG5/12/16L complex. However, the LAP process is mainly activated by phagocytosis of stimuli for TLR2 and Dectin-1 during fungal infections, Fc receptor mediated up-take of immune complexes or by TIM4 mediated phagocytosis of apoptotic debris (1).
15. Electroporation of dendritic cells can result in up to 30-40% of cell death. Therefore it is mandatory to control for cell viability and maturation phenotype of your DCs prior to any functional assay. Alternatively if available the use of the Neon™ transfection system from Invitrogen is less aggressive to the cells and result in significantly less mortality of DCs post transfection.

Acknowledgments

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References

1. Ligeon LA, Romao S, Münz C. 2017. Analysis of LC3-Associated Phagocytosis and Antigen Presentation. *Methods Mol Biol* 1519: 145-68
2. van den Hoorn T, Paul P, Jongsma ML, Neefjes J. 2011. Routes to manipulate MHC class II antigen presentation. *Curr Opin Immunol* 23: 88-95
3. Sijts EJ, Kloetzel PM. 2011. The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell Mol Life Sci* 68: 1491-502
4. Münz C. 2009. Enhancing immunity through autophagy. *Annu Rev Immunol* 27: 423-9
5. Massey AC, Zhang C, Cuervo AM. 2006. Chaperone-mediated autophagy in aging and disease. *Curr Top Dev Biol* 73: 205-35
6. Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, Potolicchio I, Nieves E, Cuervo AM, Santambrogio L. 2011. Microautophagy of cytosolic proteins by late endosomes. *Dev Cell* 20: 131-9
7. Levine B, Mizushima N, Virgin HW. 2011. Autophagy in immunity and inflammation. *Nature* 469: 323-35
8. Yang Z, Klionsky DJ. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 22: 124-31
9. Mizushima N, Yoshimori T, Ohsumi Y. 2010. The Role of Atg Proteins in Autophagosome Formation. *Annu Rev Cell Dev Biol*
10. Nakatogawa H, Ichimura Y, Ohsumi Y. 2007. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* 130: 165-78
11. Kirkin V, McEwan DG, Novak I, Dikic I. 2009. A role for ubiquitin in selective autophagy. *Mol Cell* 34: 259-69
12. Münz C. 2011. Macroautophagy during Innate Immune Activation. *Front Microbiol* 2: 72
13. Romao S, Münz C. 2011. Autophagy of pathogens alarms the immune system and participates in its effector functions. *Swiss Med Wkly* 141: w13198

14. Münz C. 2011. Antigen processing by macroautophagy for MHC presentation. *Front Immunol* 2: 1
15. Schmid D, Pypaert M, Münz C. 2007. MHC class II antigen loading compartments continuously receive input from autophagosomes. *Immunity* 26: 79-92
16. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM. 2008. Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res* 68: 6889-95
17. Uhl M, Kepp O, Jusforgues-Saklani H, Vicencio JM, Kroemer G, Albert ML. 2009. Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific $CD8^+$ T cells. *Cell Death Differ* 16: 991-1005
18. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, Mizushima N, Grinstein S, Iwasaki A. 2010. In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* 32: 227-39
19. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Muller M, Kreymborg K, Altenberend F, Brandenburg J, Kalbacher H, Brock R, Driessen C, Rammensee HG, Stevanovic S. 2005. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc Natl Acad Sci U S A* 102: 7922-7
20. Nedjic J, Aichinger M, Emmerich J, Mizushima N, Klein L. 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455: 396-400
21. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, Münz C. 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 307: 593-6
22. Leung CS, Haigh TA, Mackay LK, Rickinson AB, Taylor GS. 2010. Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. *Proc Natl Acad Sci U S A* 107: 2165-70
23. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr., Eissa NT. 2009. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med* 15: 267-76

24. Russmann H, Panthel K, Kohn B, Jellbauer S, Winter SE, Garbom S, Wolf-Watz H, Hoffmann S, Grauling-Halama S, Geginat G. 2010. Alternative endogenous protein processing via an autophagy-dependent pathway compensates for Yersinia-mediated inhibition of endosomal major histocompatibility complex class II antigen presentation. *Infect Immun* 78: 5138-50

Figure legends

Figure 1: The mRFP-GFP-LC3 reporter construct was used to monitor autophagosomes turnover. Lysosomal turnover of autophagosomes was impaired in influenza virus MP2 transfected cells, and autophagosomes did not colocalize with MHC class II loading compartment after influenza A virus infection.

A) The tandem reporter construct mRFP-GFP-Atg8/LC3 was transiently transfected into influenza A virus matrix protein 2 (MP2) or nucleoprotein (NP) transfected A549 human lung epithelial cells. GFP (sensitive to acidification and lysosomal degradation) and RFP fluorescence (insensitive to acidification and lysosomal degradation) of the reporter construct were analyzed by fluorescence microscopy. DAPI was used to stain nuclear DNA. Scale bar: 60 μm. One of three experiments is shown.

The GFP moiety of the tandem construct is sensitive to lysosomal proteolysis and quenching in acidic pH, while the mRFP is not. Therefore, the green fluorescent component of the composite yellow fluorescence for the mRFP-GFP-LC3 reporter was lost upon autophagosome fusion with lysosomes. This fluorescence change from yellow to red can be used to visualize lysosomal proteolysis and localization in acidified compartments of macroautophagy targeted GFP.

In NP transfected lung epithelial cells few yellow autophagosomes, but a high number of mRFP positive autolysosomes could be detected after transient transfection of mRFP-GFP-Atg8/LC3 .

In MP2 transfected cells in contrast, there is an accumulation of mRFP and GFP double positive vesicles, especially in the perinuclear region, suggesting impaired autophagosomes fusion with lysosomes.

B) HLA-DR4^{+} HEK 293 cells were infected with influenza A virus for 24 hours, cells were then fixed and stained with antibodies specific for MHC class II loading compartments (in the upper panel LAMP2). Autophagosomes were excluded from LAMP2 positive compartments.

Figure 2: MHC class II presentation assay using LC3 targeted antigen: example of NY-ESO-LC3

A) NY-ESO-1 specific clonal $CD4^+$ T cells were co-cultured at various effector to target cell (E:T=5:1, black bars; E:T=10:1, grey bars; E:T=15:1, white bars) ratios with the M199 melanoma cell line transfected with either NY-ESO-1, or NY-ESO-1-LC3 constructs. The next day, IFN-γ was measured in culture supernatants by ELISA to assess antigen presentation of NY-ESO-1 on MHC class II molecules. MHC class II presentation of the cognate NY-ESO-1 epitope was significantly enhanced by the Atg8/LC3 fusion. Error bars indicate standard deviations. One of three experiments is shown. Peptide pulsed targets (striped bars) and non-pulsed targets (far right) were used as positive and negative controls.

Figure 3: Silencing macroautophagy in monocyte-derived dendritic cells

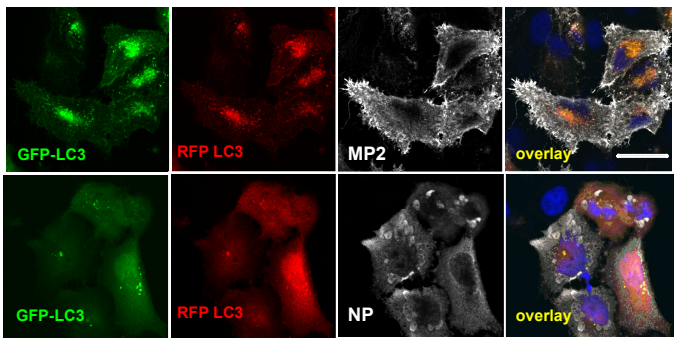
Immunofluoresence analysis of autophagosome accumulation (LC3) in immature DCs and influenza virus-infected DCs pre-treated with Atg16L1 siRNA or control scrambled siRNA: Immature DCs were pretreated at day 4 with siRNA against Atg16L1 or with a control siRNA. At day 5, cells were infected with 0.1 HA unit of influenza A virus (PR8). At day 6, cells were harvested and fixed for immunostaining with a LC3 specific antibody. Upon treatment with Atg16L1 siRNA the accumulation of autophagosomes after influenza A virus infection was down-regulated. One experiment out of 2 is shown. Scale bars: 10μm.

Figure 1

A

MP2 transfected

NP transfected



B

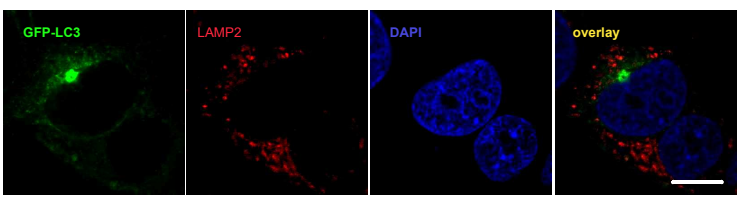


Figure 2

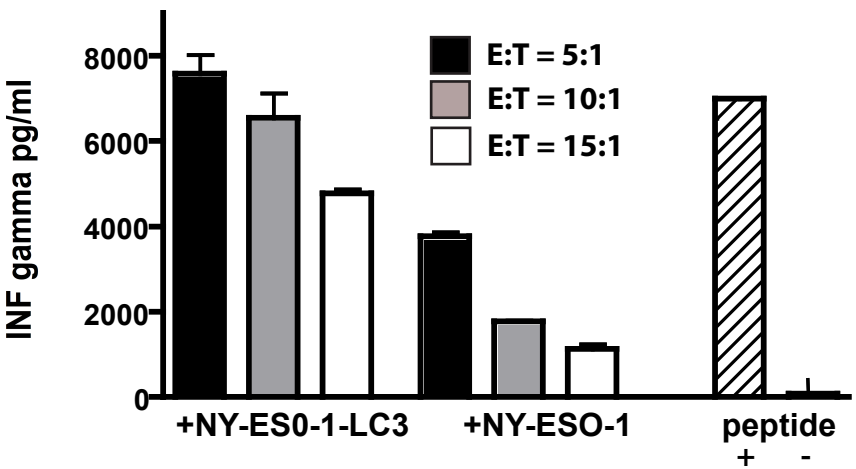
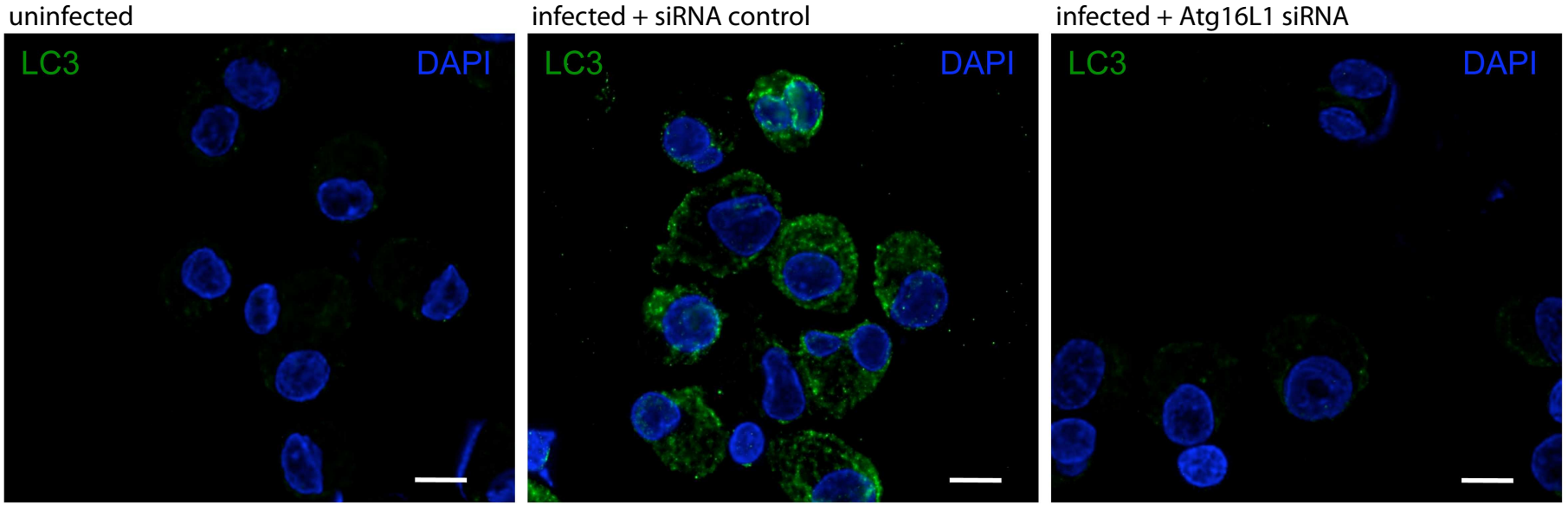
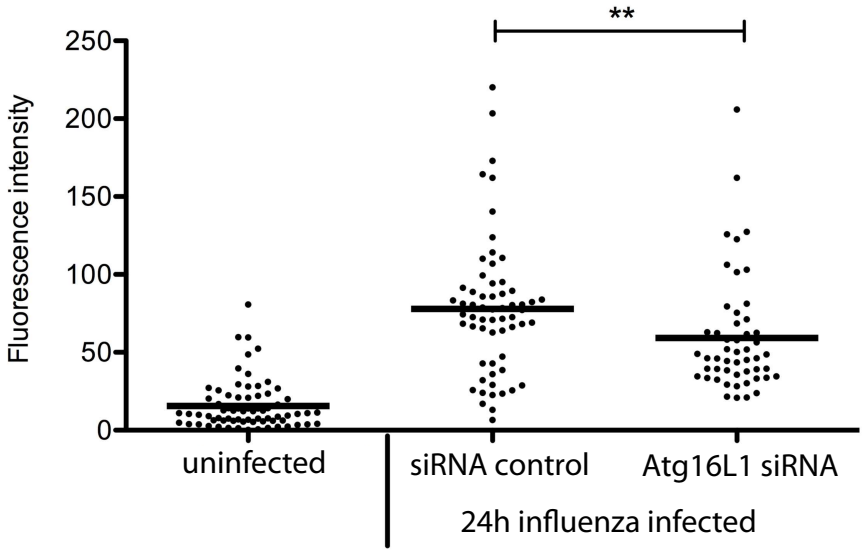

INF gamma pg/ml
8000
6000
4000
2000
0
E:T = 5:1
E:T = 10:1
E:T = 15:1
+NY-ES0-1-LC3
+NY-ESO-1
peptide
+
-

Figure 3


uninfected
infected + siRNA control
infected + Atg16L1 siRNA
LC3
DAPI
LC3
DAPI
LC3
DAPI


**
250
200
150
100
50
0
Fluorescence intensity
uninfected
siRNA control
Atg16L1 siRNA
24h influenza infected